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Original Article

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Novel antifungal activity of oligostyrylbenzenes compounds on *Candida tropicalis* biofilms

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Abstract

As sessile cells of fungal biofilms are at least 500-fold more resistant to antifungal drugs than their planktonic counterparts, there is a requirement for new antifungal agents. Olygostyrylbenzenes (OSBs) are the first generation of poly(phenylene)vinylene dendrimers with a gram-positive antibacterial activity. Thus, this study aimed to investigate the antifungal activity of four OSBs (1, 2, 3, and 4) on planktonic cells and biofilms of *Candida tropicalis*. The minimum inhibitory concentration (MIC) for the planktonic population and the sessile minimum inhibitory concentrations (SMIC) were determined. Biofilm eradication was studied by crystal violet stain and light microscopy (LM), and confocal laser scanning microscopy (CLSM) was also utilized in conjunction with the image analysis software COMSTAT. Although all the OSBs studied had antifungal activity, the cationic OSBs were more effective than the anionic ones. A significant reduction of biofilms was observed at MIC and supraMIC50 (50 times higher than MIC) for compound 2, and at supraMIC50 with compound 3. Alterations in surface topography and the three-dimensional architecture of the biofilms were evident with LM and CLSM. The LM analysis revealed that the *C. tropicalis* strain produced a striking biofilm with oval blastospores, pseudohyphae, and true hyphae. CLSM images showed that a decrease occurred in the thickness of the mature biofilms treated with the OSBs at the most effective concentration for each one. The results obtained by microscopy were supported by those of the COMSTAT program. Our results revealed an antibiofilm activity, with compound 2 being a potential candidate for the treatment of *C. tropicalis* infections.

Lay Summary

This study aimed to investigate the antifungal activity of four OSBs (1, 2, 3, and 4) on planktonic cells and biofilms of *Candida tropicalis*. Our results revealed an antibiofilm activity, with compound 2 being a potential candidate for the treatment of *C. tropicalis* infections.

Key words: Oligostyrylbenzenes, *Candida tropicalis*, biofilms, planktonic cells, antibiofilm activity.

Abbreviations

AmB	Amphotericin B
AMP	antimicrobial peptides
BBU	biofilm biomass units
CAMPs	cationic antimicrobial peptides
CFU	colony-forming unit
CLSM	confocal laser scanning microscopy
CV	crystal violet
DMSO	dimethylsulfoxide
ECM	extracellular matrix
FBS	Fetal Bovine Serum

LM	light microscope
MFC	minimum fungicidal concentration
MIC	minimum inhibitory concentration
NCPF	National Collection of Pathogenic Fungi
OD	optical density
OSBs	olygostyrylbenzenes
RPMI	Roswell Park Memorial Institute
SD	standard deviation
SDA and SDB	Sabouraud dextrose agar or broth
SMIC	sessile minimal inhibitory concentration
subMIC10	concentration 10 times lower than the MIC
supraMIC50	concentration 50 times higher than the minimum inhibitory concentrations
supraMIC100	concentration 100 times higher than the minimum inhibitory concentrations

Introduction

Some previous studies have described an increase in fungal superficial infections and serious invasive candidiasis closely related to *Candida tropicalis*.[1–3] Concerning this, biofilms are considered to be self-protective mechanisms for the growth of microorganisms and crucial to the development of chronic infections.[4,5] They have also been observed as adherent sessile cell populations attached to a surface within a slimy extracellular matrix (ECM). The sessile cells adhere to the animate or inanimate surfaces, grow and divide to form microcolonies, and finally form macrocolonies, which lead to mature biofilms.[6] The biofilms associated with *Candida* infections are clinically relevant due to their multifactorial resistance and tolerance to antifungal agents.[2,7–9] Of these, *C. tropicalis* has been reported to have a high capacity to form biofilms with a dense network of yeast and filamentous cells within an ECM.[10–12] The diffusion within *C. tropicalis* biofilms is less than that of *Candida glabrata* or *Candida krusei* biofilms.[13] This different composition and quantity of ECM permits less penetration, for example, to antifungal agents such as amphotericin B (AmB).[9,14]

An increase in biofilm resistant infections caused by *Candida* strains has stimulated research into new chemotherapeutic agents,[14,15] with it having been described that biofilms are at least 500-fold more resistant to antifungal agents than their planktonic cells.[8,15] For this reason, a constant research effort is needed to search for new antifungal agents to treat relevant infections, especially those associated with the formation of biofilms. Recently, oligostyrylbenzenes (OSBs) have emerged as a new family of compounds with antibacterial properties.[16] OSBs are simple aromatic systems with a rigid conjugate scaffold, and have been reported as first generation dendrimers of polyphenylene-vinylene (Fig. 1).[17] These have been extensively studied for their good fluorescence properties, and recently reported as being potent Gram-positive antibacterial agents.[16,18–20] However, these structures present few advantages, as OSBs have a rigid core and the functional groups are placed at a specific location. In addition, although the conjugated scaffold has no activity, the specific structure and geometry, along with the peripheral groups and the molecular polarity, make it easy to establish a correlation between the structure and activity. Nevertheless, increasing the number of active functional groups does not always result in a higher activity, because a perfect balance between the hydrophobic and hydrophilic parts of the molecule is required. The conjugated core has a high fluorescence that can be used as a probe to localize and rationalize the mechanism of these molecules. Moreover, OSBs have shown low cytotoxicity against COS-1 and VERO cells.[16] However, there is no information available about OSB antifungal action, either on planktonic cells or biofilms.

The present study aimed at evaluating the potential antifungal activity on planktonic yeast cells and the antibiofilm action of four OSBs against *C. tropicalis*.

Our results showed that compound 2 had an important fungicidal action with biofilm eradication. This OSB compound could therefore be a potential candidate for the treatment of *C. tropicalis* infections.

Methods

Culture medium and reagents

Sabouraud dextrose broth (SDB) and agar (SDA, Difco, Detroit, MI, USA), crystal violet (CV, Anedra Buenos Aires, Argentina), AmB, Roswell Park Memorial Institute (RPMI) medium, phosphate-buffered saline, Calcofluor-White M2R, Evans Blue (Sigma Aldrich Co., St. Louis, MO, USA), fetal bovine serum (FBS, Greiner Bio-One, Frickenhausen, Germany), and dimethylsulfoxide (DMSO, Merck Darmstadt, Germany).

Oligostyrylbenzene derivatives

Four OSBs with different functional groups designated 1, 2, 3, and 4 were used (Fig. 1), prepared according to standardized techniques in organic chemistry and characterized by nuclear magnetic resonance, Fourier-transform infrared spectroscopy and the electromagnetic spectrum.[16,20] For biological experiments, a stock solution of each compound (10 mg/ml) was prepared with 1% DMSO.

Yeast strain

The yeast strain *C. tropicalis* 3111 from the National Collection of Pathogenic Fungi (NCPF, Bristol, UK) was used. This strain was frozen (−80°C) in SDB with glycerol 15% (v/v). Yeast cells were subcultured onto SDA and incubated at 37°C for 24 hours for antifungal activity experiments.[21,22]

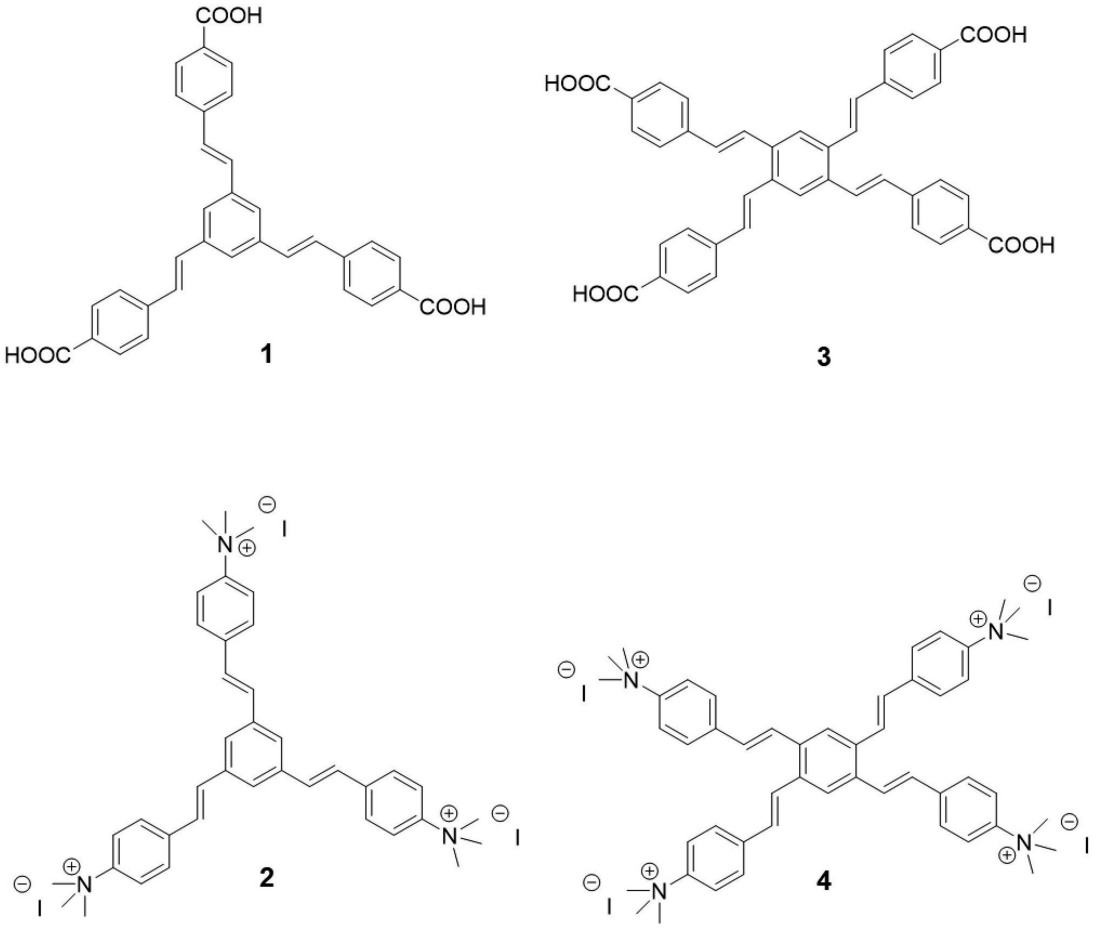

Figure 1. Chemical structure of four OSBs differently functionalized called 1, 2, 3, and 4.

Antifungal activity in planktonic yeast cells

The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) for planktonic yeast cells for each OSB compound and for AmB were determined following standard methods.[23] The MIC is defined as the lowest concentration that will inhibit visible growth of a microorganism, and the MFC is the lowest concentration that will prevent the growth of yeast after subculture onto antifungal free media.

The effects of OSBs 1, 2, 3, and 4 on biofilms were analyzed using an overnight culture in SDB with a yeast concentration of 1×10^6 colony-forming units (cfu) per ml, equal to 0.5 on the McFarland scale. RPMI medium with 0.2% glucose, glutamine and buffered with morpholino propane sulphonic acid (0.16 M , pH 7.0 $\pm$ 0.1) was used. Serial dilutions of each compound in this medium (2–256 μg/ml) were placed in a 96-well microplate in triplicate. Fungal inoculum (1×10^6 cfu/ml) was diluted in RPMI medium (1:1000) and incubated at 37°C for 48 hours.[23–26]

For the MFC experiment, we seeded each well in which fungal growth was not observed with 100 μl SDA. The broth dilutions were streaked onto SDA and incubated for 48 hours, and the lowest value of each compound which eliminated 99.9% of the initial inoculum was defined as the MFC.[27]

Antibiofilm activity

The sessile minimum inhibitory concentration (SMIC) was determined as described below. Briefly, 100 μl per well of each compound was added and the following four different concentrations were evaluated: subMIC concentration (10 times lower than MIC), MIC concentration, and two supraMIC concentrations (50 and 100 times higher than MIC). In addition, wells without AmB or OSB but with 1% (v/v) DMSO were used as control. After inoculation, the 96-well microplate was incubated for 48 hours at 37°C, and the optical density (OD) was determined at 595 nm.

The SMIC50 and SMIC80 values were defined as the values at which the OD values decreased by 50 and 80%, respectively.[26,27] In the case of AmB, 200 μg/ml corresponded to the SMIC80 value. The biofilms were measured by staining the adherent sessile cells with CV on a flat-bottomed 96-well microplate (Greiner Bio-One).[24–29] Using the 1.0 McFarland standards, yeast cells were placed into wells and incubated for 90 minutes at 37°C. Previously, plates had been pretreated with FBS 50% (v/v) to increase the adherence of the yeast cells to the well surfaces. The supernatant was removed, and the microplate was washed. Then, 200 μl of SDB was added and incubated for 48 hours at

37°C without shaking. After this incubation time, nonadhered cells were washed, and 200 μl of each compound was added and incubated for 48 hours at 37°C. The samples were gently rinsed and allowed to dry for 24 hours at room temperature prior to CV staining. Yeast cells were washed with phosphate-buffered saline to pH 7.0 ± 0.1. The microplate was stained with 200 μl of 1% (w/v) CV for 5 minutes, after which the wells were washed with phosphate-buffered saline to remove unreacted dye, and then 200 μl of ethanol/glacial acetone bleaching solution (70/30) were added. The total biomass of biofilms was quantified by spectrophotometric reading of the OD at 595 nm using a microplate reader (Infinite F50 Model, Tecan, Australia). The average OD of the control wells (ODc, containing only SDB at pH 6.5) was subtracted from the OD of all wells tested. The untreated biofilms were considered to be a positive control of mature biofilm formation, and were treated with AmB (200 μg/ml) as a positive control of antifungal activity on mature biofilms.[24–26]

The biofilm biomass unit (BBU) is an arbitrary number that is related to the optical density of the biofilm.[29] The percentage of biofilm reduction was calculated using the following equation:[30]

$$\%R = \frac{\text{Control OD}_{595\text{ nm}} - \text{OSB OD}_{595\text{ nm}}}{\text{Control OD}_{595\text{ nm}}} \times 100$$

Cultivable sessile yeast cells

Cultivable sessile cells of the mature biofilms were determined after exposure to the compounds by plate count (cfu/ml). The incubation time was 48 hours at 37°C without shaking. The supernatant wells were removed and 100 μl of sterile phosphate-buffered saline were added, after which, the microplate was sonicated (40 kHz, 60 seconds) in order to resuspend and homogenize the sessile cells. Then, 100 μl of sterile phosphate-buffered saline previously diluted (1/1000) in SDB were seeded onto a SDA plate and incubated for 24–48 h at 37°C.[31] Finally, correlation studies between cfu/ml and BBU were peformed.[24–26]

Microscopic analysis of biofilms

C. tropicalis biofilms were developed on glass covers (12 mm Ø, Menzel Deckgläser, Braunschweig, Germany) placed in a 24-well microplate (Greiner Bio-One) treated with FBS 50% (v/v). After biofilm compound exposure, the supernatants were removed, and the covers were rinsed with sterile phosphate-buffered saline. The samples studied at the highest concentration of eradication were the positive control of mature biofilms (without antifungal treatment) and the samples with compounds or with AmB.[5,24–26]

To carry out the light microscope (LM) analyses, the samples were washed and dried at 24°C. Then, each condition was stained with 1% (w/v) CV for 5 minutes, and the samples were observed at 100–600× using an inverted LM (Ax overt 40 C, Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Finally, morphology yeast forms and the biofilm micro- and macrocolonies were observed.[4,26]

Table 1. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of four oligostyrylbenzenes compounds (1, 2, 3, and 4) and amphotericin B (AmB) evaluated in planktonic cells of *Candida tropicalis*.

	1 (μg/ml)	2 (μg/ml)	3 (μg/ml)	4 (μg/ml)	AmB (μg/ml)
MIC	32	8	64	16	0.25
MFC	64	8	128	16	0.25

Biofilms were further characterized by confocal laser scanning microscopy (CLSM), with the biofilms being stained with Calcofluor-White 15 μl in a 1:1 mixture of Calcofluor-White (0.5 g/l) and 10% (w/v) KOH, which was added to each of the glass covers and incubated for 5 minutes in the dark at room temperature.[4,22] Afterward, the samples were assembled to perform the analysis. The covers were examined by using a Fluoview FV1000 Spectral Olympus CSLM (Olympus Latin America, Miami, FL, USA) equipped with a PLAPON 60X O NA:1.42 Olympus oil immersion lens, with static CLSM images being acquired at 0.5 – 1 μm z intervals.[21,24–26]

For microscopic analyses, three independent experiments were performed in triplicate. These images were obtained and evaluated independently by two investigators (M.A.Q. and M.G.P.). Representative microscopic images and statistical results are shown in Figure 3. An analysis was performed using the software COMSTAT.[32,33] The following variables that describe the structure of biofilms were analyzed: biomass or biovolume ($\mu m^3/\mu m^2$), average microcolony volume (μm^3), maximum diffusion distance (μm), average diffusion distance (μm) and roughness coefficient.[32,33] The evaluation of the images was carried out using the ImageJ free software.[21,25,26,31]

Statistical analysis

The data represent the mean (± standard deviation [SD]) of three independent experiments, which were performed in triplicate. The relationship between the CV and cfu/ml assay values was calculated using the Pearson product correlation. The data were also analyzed by using analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons. Values of $^*P < .01$ and $^{**}P < .001$ were considered to be significant when comparing with nontreated biofilms.

Results

To study the relationship between antifungal action and antibiofilm activity, the MIC and MFC values of each compound against planktonic fungal cells were determined. Table 1 summarizes the MIC and MFC determinations of *C. tropicalis* NCPF 3111 in relation to the OSBs. The lowest value of MFC that killed 99.9% of yeast was for compound 2, which achieved the fungicidal endpoint with 8 μg/ml. Compound 4 also revealed

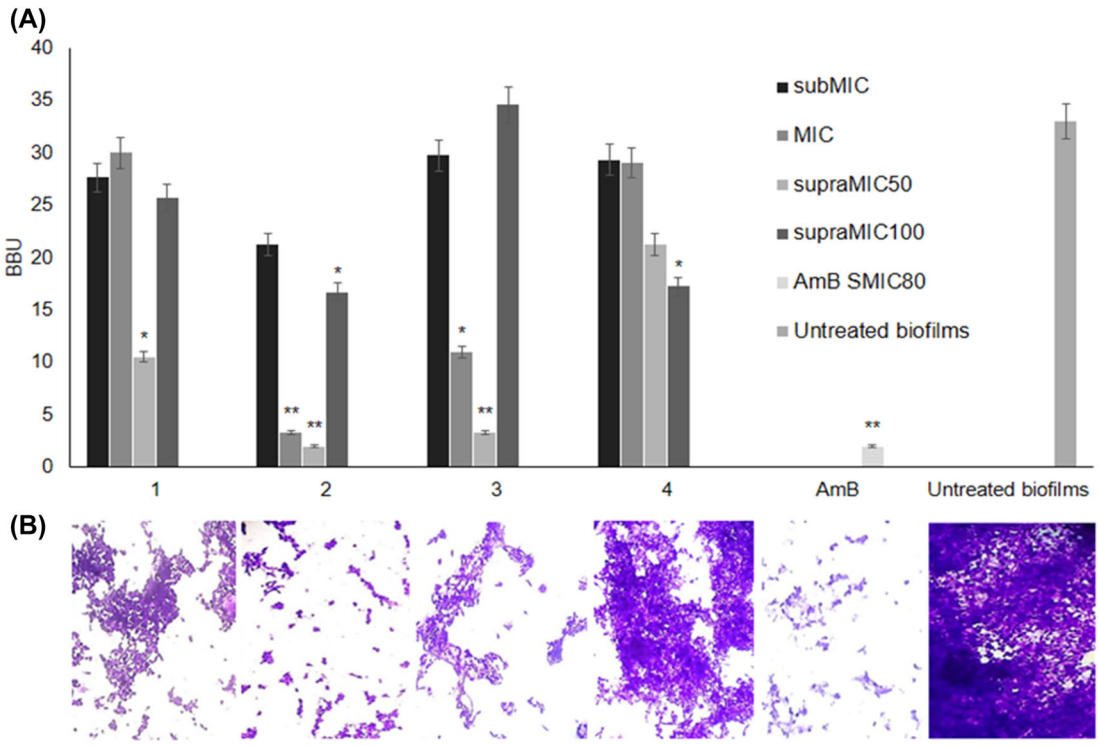

Figure 2. Antifungal activity of oligostyrylbenzene (OSB) derivatives and Amphotericin B (AmB) on *Candida tropicalis* biofilms. (A) Quantification by crystal violet (CV) staining expressed in biofilm biomass units (BBU). (B) The samples were stained with CV for LM (100x). Abbreviations: MIC, minimum inhibitory concentration; subMIC10, concentration 10 times lower than the MIC value; supraMIC50, concentration 50 times higher than the MIC value; supraMIC100, concentration 100 times higher than the MIC value; AmB SMIC80, 80% reduction in the BBU of the biofilms treated with the antifungal compared with control wells corresponding to supraMIC800. All experiments were performed in triplicate, and the numerical data were presented as means ± standard deviation. Differences in $*P < .01$ and $**P < .001$ were considered significant compared with untreated biofilms.

a good fungicidal activity (16 μg/ml). With respect to AmB, the MIC and MFC values were 0.25 μg/ml. The MICs were close to MFCs for the four OSBs, indicating a fungicidal effect on planktonic yeast cells. Nevertheless, different values of MICs were obtained for each compound.

Candida species have been reported as being potent biofilm producers. This finding was studied using a microtiter plate biofilm assay and CV stains for *C. tropicalis* NCPF 3111, a strain classified as a prolific biofilm former (BBU 33.00 ± 0.60). Under subMIC10 concentrations, the four compounds produced little reduction in the BBU. However, the BBU decreased significantly at MIC values for compound 2 (3.27 ± 0.81 $**P < .001$). This value was almost 10 times lower than that found for untreated biofilms, which represented a reduction of 90% ($**P < .001$) (Fig. 2A). In contrast, supraMIC50 enhanced the effect of compounds 2 and 3, reducing the BBU. Furthermore, this reduction for compound 2 represented an eradication rate of 94% ($**P < .001$), which was similar to the AmB (200 μg/ml) value. In contrast, the biofilm reduction was lower at the supraMIC100 value for all OSBs, possibly due to the paradoxical effect, which also was reported for other antifungal agents. It should also be noted that during the experiment the negative control (with DMSO) did not show any activity. In addition, a good correlation between the CV assay and cfu/ml was obtained (data not shown). The LM images indicated the presence of yeast, pseudo- and true hyphae, and micro- and macrocolonies in untreated biofilms. We confirmed antibiofilm activity, which was exhibited by a decrease in the thickness of the biofilms. The presence of biofilm microcolonies of compound 2 was similar to that of AmB, in terms of having the highest levels of reduction (Fig. 2 B).

The biofilm architecture was measured using CLSM, which can be used to obtain information about the topography and biofilm organization, as well as the sessile cell morphological details and spatial localization. Figure 3 shows the CLSM images for XY (top) and XZ (bottom), with the blue channel showing Calcofluor-White dying sessile cell walls of *C. tropicalis,* and with CLSM confirming the antibiofilm activity of OSBs on mature biofilms. Figure 3A–D displays the CLSM images, which reveal a decrease in the mature biofilm thickness when treated with OSBs at the most effective concentration for both AmB (SMIC80) (Fig. 3E) and untreated biofilms (Fig. 3F). The three-dimensional images of the topographic surfaces and architecture of the biofilms reveal void space in the matrix, channels, and pore morphology alterations, which could change in stream velocity inside the ECM (Fig. 3, inset).

Various biofilm morphological parameters under different treatment strategies were quantified using the software COMSTAT (Table 2). The highest reduction of biomass was obtained for OSB compound 2, compared to the untreated biofilms and AmB. The average microcolony volume was reduced in these

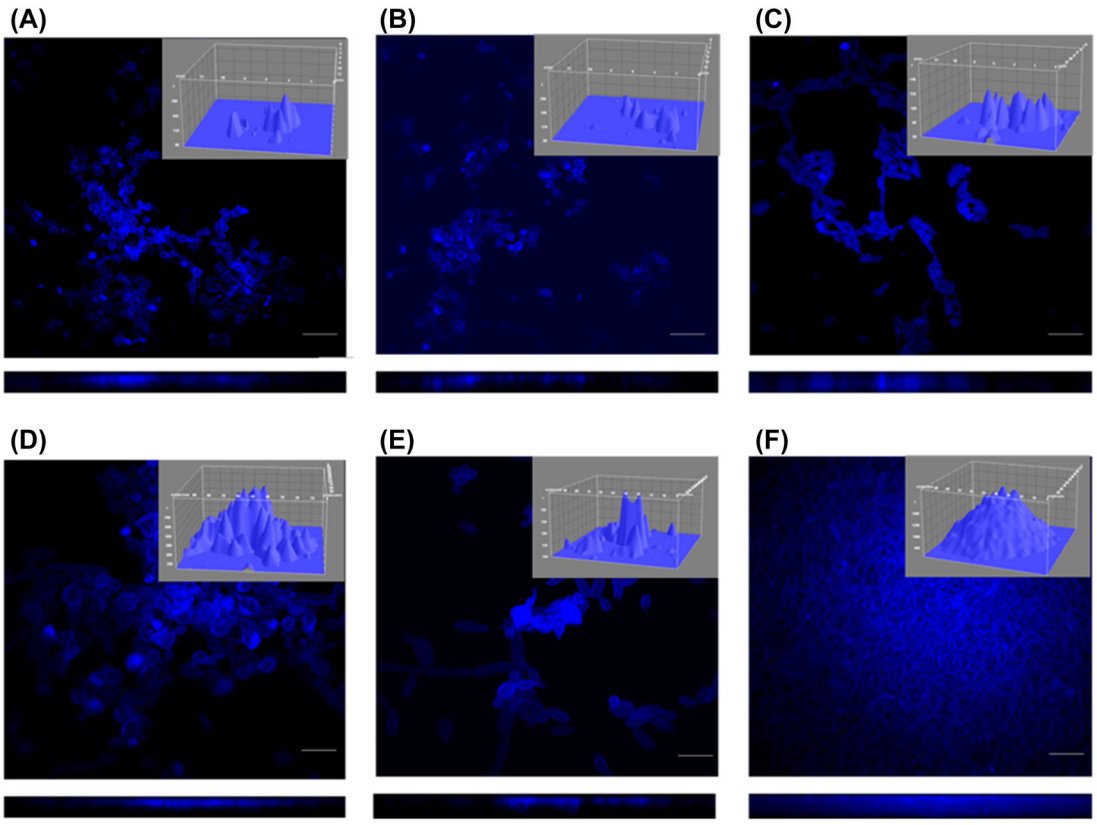

Figure 3. Confocal laser scanning microscopy (CLSM) images showing the effect of oligostyrylbenzene (OSB) compounds on *Candida tropicalis* biofilms. (A) 1 at supraMIC50, (B) 2 at MIC, (C) 3 at supraMIC50, (D) 4 at supraMIC100, (E) AmB at SMIC80, and (F) untreated biofilms. The blue channel shows Calcofluor-White dying sessile cell walls. Images were captured at 600x magnification and a scale bar of 10 μm. Inset: Different topographic surface architectures of mature biofilm obtained by 3D image structure reconstruction.

Table 2. COMSTAT analysis of architectural parameters of *Candida tropicalis* mature biofilms treated with four oligostyrylbenzenes (OSBs) compounds or with amphotericin B (AmB).

	1 (supra MIC50)	2 (supra MIC50)	3 (supra MIC50)	4 (supra MIC50)	AmB (SMIC80)	Untreated biofilms
Bio-volume ($\mu m^3/\mu m^2$)	1.35 ± 0.24**	0.57 ± 0.01**	1.48 ± 0.29**	1.48 ± 0.31**	0.76 ± 0.07**	9.27 ± 1.61
Average microcolony volume (μm^3)	16.70 ± 0.32**	6.09 ± 0.14**	12.95 ± 0.55*	11.55 ± 0.13	9.03 ± 0.20	10.85 ± 0.74
Maximum diffusion distance (μm)	9.65 ± 0.12	17.19 ± 1.28**	17.01 ± 1.77**	8.12 ± 0.43	14.29 ± 0.65***	9.60 ± 0.40
Average diffusion distance (μm)	0.51 ± 0.02*	0.81 ± 0.03**	0.29 ± 0.08	0.16 ± 0.01*	0.12 ± 0.01*	0.25 ± 0.05
Roughness coefficient	1.39 ± 0.19**	1.83 ± 0.11**	1.77 ± 0.03**	1.30 ± 0.24**	1.62 ± 0.01**	0.17 ± 0.01

All experiments were performed in triplicate, and numerical data were presented as means ± standard deviation. *$P < .01$ and **$P < .001$ values were considered significant when compared with nontreated biofilms.

OSBs, as illustrated in the LM image. In addition, biofilms treated with compound 2 exhibited a higher maximum diffusion distance, as well as a greater average diffusion distance and roughness coefficient, compared to untreated biofilms.

Discussion

Although non-*albicans Candida* species have emerged as an important cause of infections, there is still a lack of information about their antifungal resistance and pathogenicity.[1–3,9,10,37,38] Consequently, there is a constant need for new research into novel antifungal agents. *C. tropicalis* has emerged as one of the most important Candida species in terms of epidemiology and virulence, especially in Asia and the majority of Latin American countries.[7,37] Moreover, this species has been recognized as being a very strong biofilm producer, even greater than other *Candida* species.[1,2,10–12,15]

Few antifungal drugs can be used systemically for invasive fungal infections, and are limited when compared with other drugs to treat bacterial infections.[6–9,37–39] In addition, they are increasingly compromised by the rise of drug resistance.[6,8,14] In

particular, the development of new antibiofilm agents is urgently needed to reduce the incidence of biofilm-associated infections, due to biofilm involvement in infectious disease and the spread of multi-drug resistance.[40]

We have previously shown that these compounds presented antibacterial activity, and a preliminary study was performed to test these compounds on the bacteria.[16] The cationic OSBs 2 and 4 are bactericides and penetrated to the cell, while the anionic OSBs 1 and 3 remained stuck outside on the membrane and developed a bacteriostatic behavior. Although the mechanism of OSB molecules as antifungals is still unclear, there are some physicochemical properties of these molecules that, on comparison with others, can help elucidate their action mechanism on planktonic cells. To our knowledge, no prior studies have examined the killing activities of OSBs against *C. tropicalis*. In the present study, the compounds evaluated showed a fungicidal effect on fungal planktonic forms, with the MIC and MFC values being found to be between 32 and 256 times higher than the MIC of AmB. The OSBs have an amphiphilic structure with a hydrophobic conjugate core and peripheral ionic groups. Electrostatic interactions of OSBs with polysaccharides (negatively charged) or proteins (positively charged), and hydrophobic interaction with lipids in the fungus wall, could be the main causes of antifungal action of OSBs. In fact, the OSBs revealed different antifungal activities on planktonic cells (MICs 8–64 μg/ml), and the MIC values were close to those of the MFCs (MFC/MIC $\leq$ 2). This has also been previously observed for other molecules with similar structures, such as quaternary ammonium salts or the well-known benzalkonium chlorides.[34] Although different values of MICs were found for each compound, the antimicrobial peptides (AMP) have broad-spectrum activities and multiple mechanisms of action. Cationic antimicrobial peptides (CAMPs) are short, positively charged peptides with an amphipathic structure.[35] Concerning these, only a few studies have described the effect of CAMPs on biofilm formation by fungal pathogens such as *Candida*.[36] Similarly, the target of CAMPs is the microbial cytoplasmic membranes, and their mechanism of action is associated with pore-forming and general membrane permeabilization.[35] According to different studies, OSB antimicrobial activity depends on the electrostatic charge and the hydrophobicity of its structure.[41,42] This suggests that antifungal activity may depend on the balance between the charge and the hydrophobicity present in the OSB molecules. From our results, we hypothesize that OSB may interact with the cell wall of the fungi, as cationic OSB **2** and **4** are antifungal and revealed a higher activity than anionic OSB 1 and 3. Thus, this different behavior observed may be partly explained by the fungal cell wall being mainly composed of negatively charged polysaccharides that interact strongly with cationic OSB.

Compound 1 was active against the *C. tropicalis* NCPF 3111 biofilms, attaining SMIC50 values (which caused 50% biofilm reduction) at the supraMIC50 concentration. In addition, compound 2 reached the SMIC80 value at MIC and supraMIC50 concentrations, with compound 3 achieving SMIC50 at MIC and SMIC80 at supraMIC50 values, and the lowest biofilm reduction being observed for compound 4. Our results revealed that compound 2 was more active than the other OSBs in sessile cells, as well as in planktonic forms, since it required a concentration 50 times higher than its MIC (supraMIC50) to reach SMIC80. To produce the same effect, AmB had to be used at a concentration 800 times higher than its MIC (supraMIC800). Similarly, Tolosa et al. reported that compounds 1 and 2 were more active as antibacterials than compounds 3 and 4.[16]

At supraMIC100 values, our results demonstrated that biofilm reduction was lower in the four OSB treatments, being more susceptible to reduction at lower concentrations. This was possibly due to a paradoxical antifungal effect,[43] which has been defined as regrowth occurring at two dilutions above SMIC80 and having been described for *Candida* spp. in an *in vitro* biofilm model with echinocandins.[15,43,44] The nature of this paradoxical effect, however, is not yet fully understood and has been the focus of many studies.[45] Melo et al. analyzed the manifestation of the paradoxical effect for several isolates of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*.[46] Interestingly, paradoxical growth was more frequently observed when the isolates were grown as biofilms (80%) compared to planktonic cell culturing conditions (40%). At the same time, for echinocandin, one important factor that determines the manifestation of this paradoxical effect is the concentration itself, as demonstrated by paradoxical growth in the presence of high echinocandin concentrations.

The LM analysis showed that the *C. tropicalis* strain produced a striking biofilm with oval blastospores, pseudohyphae and true hyphae. CLSM has been used to evaluate the architecture, topographic biofilm surface and the morphological and spatial localization of sessile forms inside the biofilms. The biomass biofilm parameters, such as roughness coefficient, pores and voids inside treated biofilms, were significantly different among the different OSBs. The biofilm bio-volume ($\mu m^3/\mu m^2$), along with the average micro-colony volume (μm^3), shows the forms of the sessile cells occupying the surface. An established biofilm comprises sessile cells and ECM, which in turn, are comprised of polysaccharides, proteins, extracellular DNA (eDNA) and lipids. The biofilm architecture determines the microenvironment, because it governs the liquid and nutrient flow, charge, stability properties and hydrophilic and hydrophobic interactions.[31,32]

Fungal infections are recurrent in the clinical environment, so the development of novel approaches to treat *Candida* infections has a great clinical relevance, especially those associated with biofilms, because antifungal drugs have little effect on them. Our results revealed, for the first time, fungicidal action in planktonic yeast cells and an important antibiofilm activity

of OSB compounds. Moreover, OSB 2 could be a potential candidate for *C. tropicalis* infections.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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